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NSF ATPase and α-/β-SNAPs Disassemble the AMPA Receptor-PICK1 Complex

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Summary

AMPA receptor (AMPAR) trafficking is crucial for synaptic plasticity that may be important for learning and memory. NSF and PICK1 bind the AMPAR GluR2 subunit and are involved in trafficking of AMPARs. Here, we show that GluR2, PICK1, NSF, and α-/β-SNAPs form a complex in the presence of ATPγS. Similar to SNARE complex disassembly, NSF ATPase activity disrupts PICK1-GluR2 interactions in this complex. α- and β-SNAP have differential effects on this reaction. SNAP overexpression in hippocampal neurons leads to corresponding changes in AMPAR trafficking by acting on GluR2-PICK1 complexes. This demonstrates that the previously reported synaptic stabilization of AMPARs by NSF involves disruption of GluR2-PICK1 interactions. Furthermore, we are reporting a non-SNARE substrate for NSF disassembly activity.

Introduction

Recent studies reveal that AMPA receptor (AMPAR) trafficking is involved in synaptic plasticity (Luscher et al., 2000; Man et al., 2000; Carroll et al., 2001). Long-term potentiation (LTP) in hippocampus involves insertion of AMPARs into the synaptic membrane by exocytosis (Shi et al., 1999; Lu et al., 2001). Some forms of long-term depression (LTD) in the cerebellum (Matsuda et al., 2000; Wang and Linden, 2000) and hippocampus (Luscher et al., 1999; Beattie et al., 2000; Iwakura et al., 2001) are mediated at least in part by AMPAR endocytosis.

Proteins have been identified that bind the C terminus of GluR2 subunit, and are involved in AMPAR trafficking (Braithwaite et al., 2000; Scannevin and Huganir, 2000). The extreme C-terminal residues, -SVKI, interact with *A*MPAR *b*inding *p*rotein (ABP), *g*lutamate *r*eceptor *i*nteracting *p*rotein (GRIP), and *p*rotein *i*nteracting with *C-K*inase-*1* (PICK1) (Dong et al., 1997; Srivastava et al., 1998; Xia et al., 1999). A juxtamembrane region binds *N*-ethylmaleimide *s*ensitive *f*usion protein (NSF) (Osten et al., 1998; Nishimune et al., 1998; Song et al., 1998).

NSF is essential for membrane fusion events (Rothman, 1994; Lin and Scheller, 2000), which require the formation of a complex comprising SNARE proteins from target and vesicle membranes, bringing the two lipid bilayers close together so that fusion occurs. For subsequent rounds of fusion, the SNARE complex must be disassembled. α-SNAP binds the SNARE complex allowing binding of NSF, and ATP hydrolysis provides a driving force that dissociates the complex (Sollner et al., 1993; Hanson et al., 1995; Hayashi et al., 1995). β-SNAP is 83% identical to α-SNAP (Whiteheart et al., 1993). Although little is known of its specialized function, β-SNAP can substitute for α-SNAP in regulated exocytosis and in vitro 20S complex assembly-disassembly (Wilson et al., 1992; Hayashi et al., 1995; Sudlow et al., 1996). Conformational modification of SNAREs and disruption of the SNARE complex are the only functions for NSF and SNAPs identified to date.

The role of NSF in AMPAR function has been studied using a peptide corresponding to the binding site on GluR2 that blocks the AMPAR-NSF interaction and results in a reduction in AMPARs at the synapse (Nishimune et al., 1998; Song et al., 1998; Noel et al., 1999; Luscher et al., 1999). This indicates that NSF maintains a high level of synaptic AMPARs, either by preventing their removal by endocytosis or facilitating their insertion by exocytosis. The same peptide treatment occludes NMDAR-dependent LTD (Luscher et al., 1999; Luthi et al., 1999) and inhibition of NSF function by N-ethylmaleimide (NEM) enhances AMPAR endocytosis (Luscher et al., 1999). This suggests that the NSF-GluR2 interaction negatively regulates LTD by inhibition of AMPAR endocytosis.

ABP/GRIP act as receptor anchors at the plasma membrane and at an intracellular site (Osten et al., 2000; Daw et al., 2000). The PICK1-GluR2 interaction is required for the expression of some forms of LTD (Xia et al., 2001; Kim et al., 2001), suggesting that PICK1 stimulates AMPAR removal from the synaptic plasma membrane by endocytosis. In support of this, NMDAR-dependent AMPAR internalization in hippocampal neurons involves GluR2-PICK1 interactions (Iwakura et al., 2001), and we have demonstrated that PICK1 overexpression results in translocation of GluR2 homomers to an intracellular compartment (Perez et al., 2001).

NSF and PICK1 are two proteins thought to have crucial but independent roles in AMPAR trafficking. These studies, plus the established role of NSF as a disassembling chaperone, suggest that NSF might regulate GluR2-PICK1 interactions. Here, we show that this is indeed the case. The ATPase activity of NSF in conjunction with SNAPs disrupts the GluR2-PICK1 interaction. α-/β-SNAP have differential effects on this activity. Consistent with this, we demonstrate that α-/β-SNAP have differential effects on AMPAR trafficking in hippocampal neurons. This indicates that disassembly of GluR2-PICK1 complexes by NSF and SNAPs plays a critical role in regulating AMPAR density at the synapse.

Results

The GluR2-PICK1 Complex Binds Higher Levels of NSF Compared to GluR2 Alone

We previously reported that the NSF-GluR2 interaction in brain is ATP sensitive (Osten et al., 1998). Here, we

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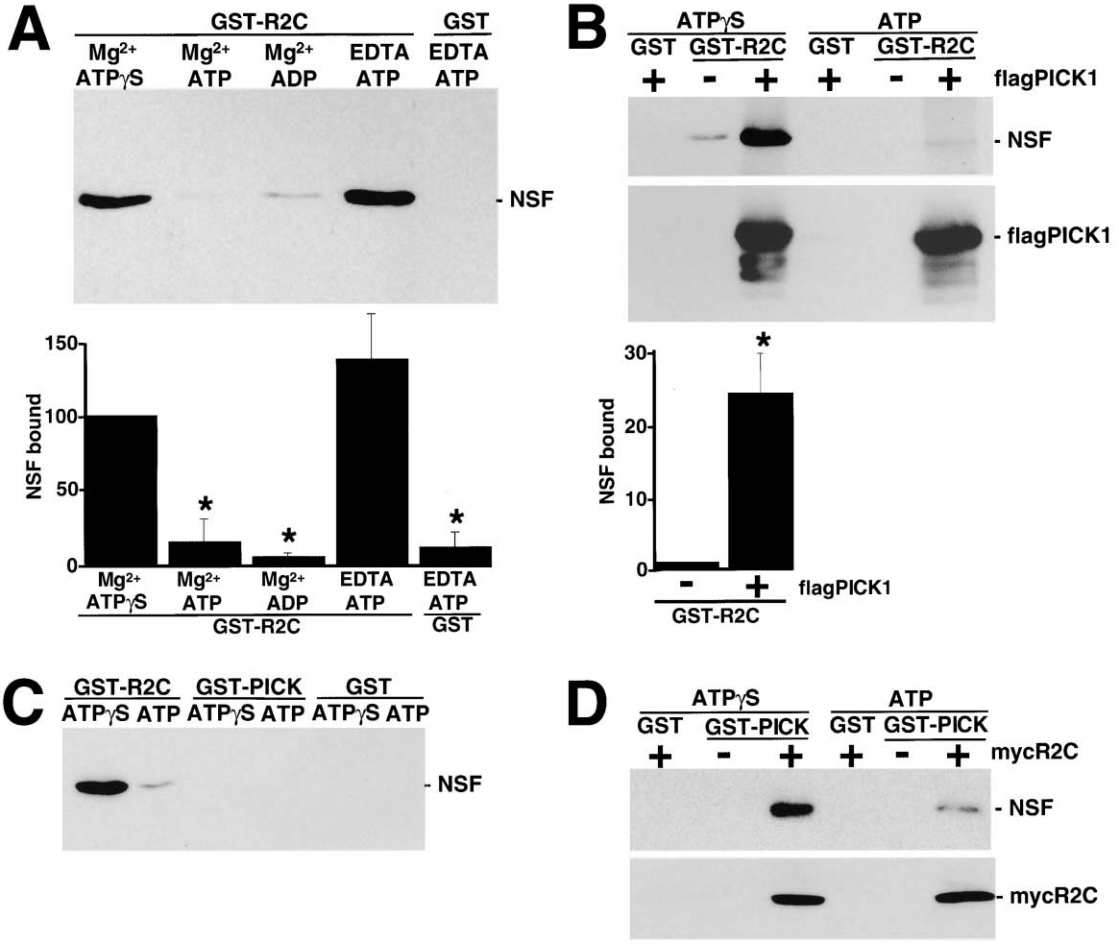

Figure 1. NSF Binds Strongly to the GluR2-PICK1 Complex In Vitro

(A) His_6NSF binding to GST-GluR2 C terminus (R2C) is sensitive to ATP hydrolysis. Five micrograms GST-R2C was immobilized on glutathione agarose beads and incubated with 10 nM his_6NSF in buffer A plus nucleotides. Beads were washed thoroughly in the same buffer, and bound NSF detected by Western blotting using anti-NSF antiserum. Top panel shows representative Western blot, bottom panel shows data from three experiments quantitated by scanning densitometry. Values are relative to MgATPγS condition. ($n = 3$, t test, $^*p < 0.05$.)

(B) NSF binds R2C-PICK1 complex more strongly than R2C alone. Two micrograms of his_6flagPICK1 was bound to 5 μg GST-R2C immobilized on glutathione-agarose beads followed by incubation with 10 nM his_6NSF in buffer A plus ATP/ATPγS. Beads were washed thoroughly in the same buffer. Bound NSF and flagPICK1 were detected by Western blotting using anti-NSF and anti-flag antibodies. Top panel shows representative Western blots, bottom panel shows data from three experiments quantitated by scanning densitometry to show enhanced NSF binding to R2C-PICK1 complex, compared to R2C alone. Values are relative to R2C alone condition ($n = 3$, t test, $^*p < 0.05$).

(C) NSF does not bind PICK1 alone. Five micrograms GST-PICK1 was immobilized on glutathione-agarose beads and incubated with 10 nM his_6NSF in buffer A plus ATP/ATPγS. The beads were washed thoroughly in the same buffer, and bound NSF detected by Western blotting using anti-NSF antiserum.

(D) R2C recruits NSF to GST-PICK1. Two micromoles of his_6mycR2C was bound to 5 μg GST-PICK immobilized on glutathione-agarose beads. These complexes were incubated with 10 nM his_6NSF in buffer A plus ATP/ATPγS. Beads were washed thoroughly in the same buffer, and bound NSF and mycR2C were detected by Western blotting using anti-NSF and anti-myc antibodies.

expressed GluR2 C terminus (R2C) as a GST fusion and NSF as a his_6 protein and analyzed the interaction of these purified proteins in the presence of nucleotides (Figure 1A). His_6NSF bound GST-R2C in Mg/ATPγS or EDTA/ATP, both of which represent nonhydrolyzable ATP. NSF is not retained by R2C in the presence of hydrolyzable ATP. In Figure 1B, his_6flag-PICK1 was pre-bound to GST-R2C followed by incubation with his_6NSF. NSF shows dramatically increased R2C binding when PICK1 is also bound. The increased NSF binding to the R2C-PICK1 complex retains sensitivity to ATP hydrolysis. To investigate the possibility that the increased NSF binding was due to a direct interaction between NSF and PICK1, we expressed GST-PICK1 and analyzed his_6NSF binding. Figure 1C shows that NSF does not bind GST-PICK1 alone. R2C was expressed as a his_6myc-tagged protein, which binds strongly to GST-PICK1 (Figure 1D). As expected, NSF shows a robust, ATP-dependent interaction with this complex. These data demonstrate that NSF-GluR2 binding is dramatically enhanced when PICK1 is present in the complex, and that NSF binding is sensitive to ATP hydrolysis.

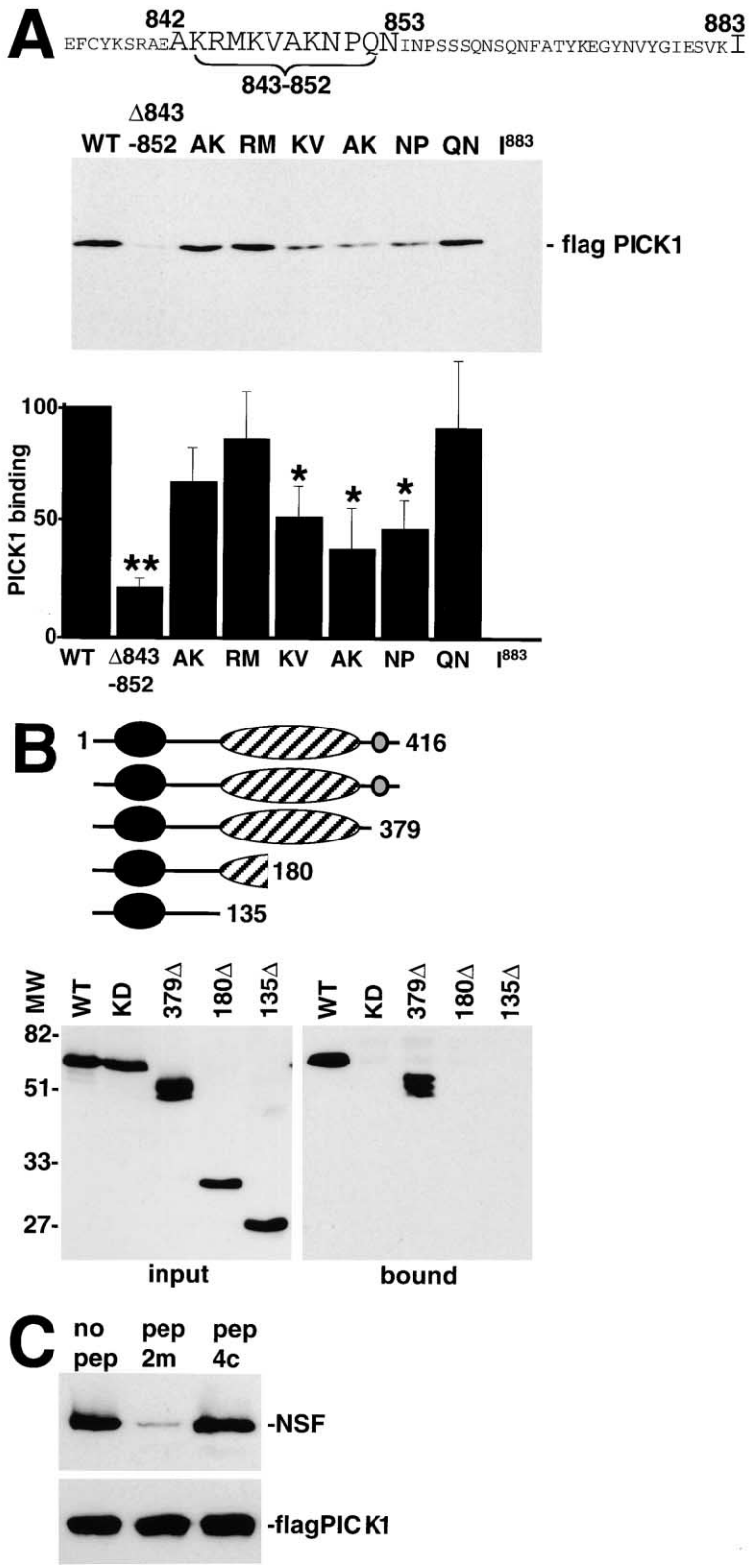

Figure 2. PICK1 Makes a Non-PDZ Contact in the "NSF Binding Region" of GluR2

(A) Mutations in the "NSF binding region" reduce PICK1 binding to GluR2. Mutations were made in GST-R2C where the residues indicated were substituted by alanine residues. An additional mutation was made by deleting the entire NSF binding site between residues 843–852. Five micrograms of the GST-R2C mutants was immobilized on glutathione beads and incubated with 10 nM his_6flag-PICK1 in buffer A lacking nucleotide. Beads were washed thoroughly and bound flag-PICK1 was detected by Western blotting using anti-flag antibody. Top panel shows representative Western blot, bottom panel shows data from six experiments quantitated by scanning densitometry. Values are relative to wild-type binding ($n = 6$, t test, $^*p < 0.05$, $^{**}p < 0.0001$).

(B) A non-PDZ domain region of PICK1 is required for binding to GluR2. Five micrograms of wild-type GST-R2C was immobilized on glutathione beads and incubated with 10 nM his_6flagPICK1 mutants as shown in top panel in buffer A lacking nucleotide. Beads were washed thoroughly and bound proteins were detected by Western blotting using anti-flag antibody (right). Relative inputs of PICK1 mutants are also shown (left).

(C) NSF binding region peptide does not interfere with R2C-PICK1 interaction. Ten nanomoles his_6NSF or his_6flagPICK1 was preincubated with 250 μM pep2m/pep4c in buffer A supplemented with ATPγS, followed by incubation with 5 μg of GST-R2C immobilized on glutathione beads in the same buffer. The beads were washed thoroughly and bound proteins were detected by Western blotting.

PICK1-GluR2 Interaction Involves the NSF Binding Region on GluR2

PICK1 is known to bind GluR2 via a PDZ interaction (Xia et al., 1999). We predicted that the strong interaction of NSF with R2C-PICK1 complexes may be due to PICK1 making contacts with GluR2 within the NSF binding region. NSF binds residues 843–852 of GluR2, with N851 and P852 especially critical (Osten et al., 1998). Binding of his_6flagPICK1 to GST-R2C mutated in this region demonstrated that the "NSF region" is also important

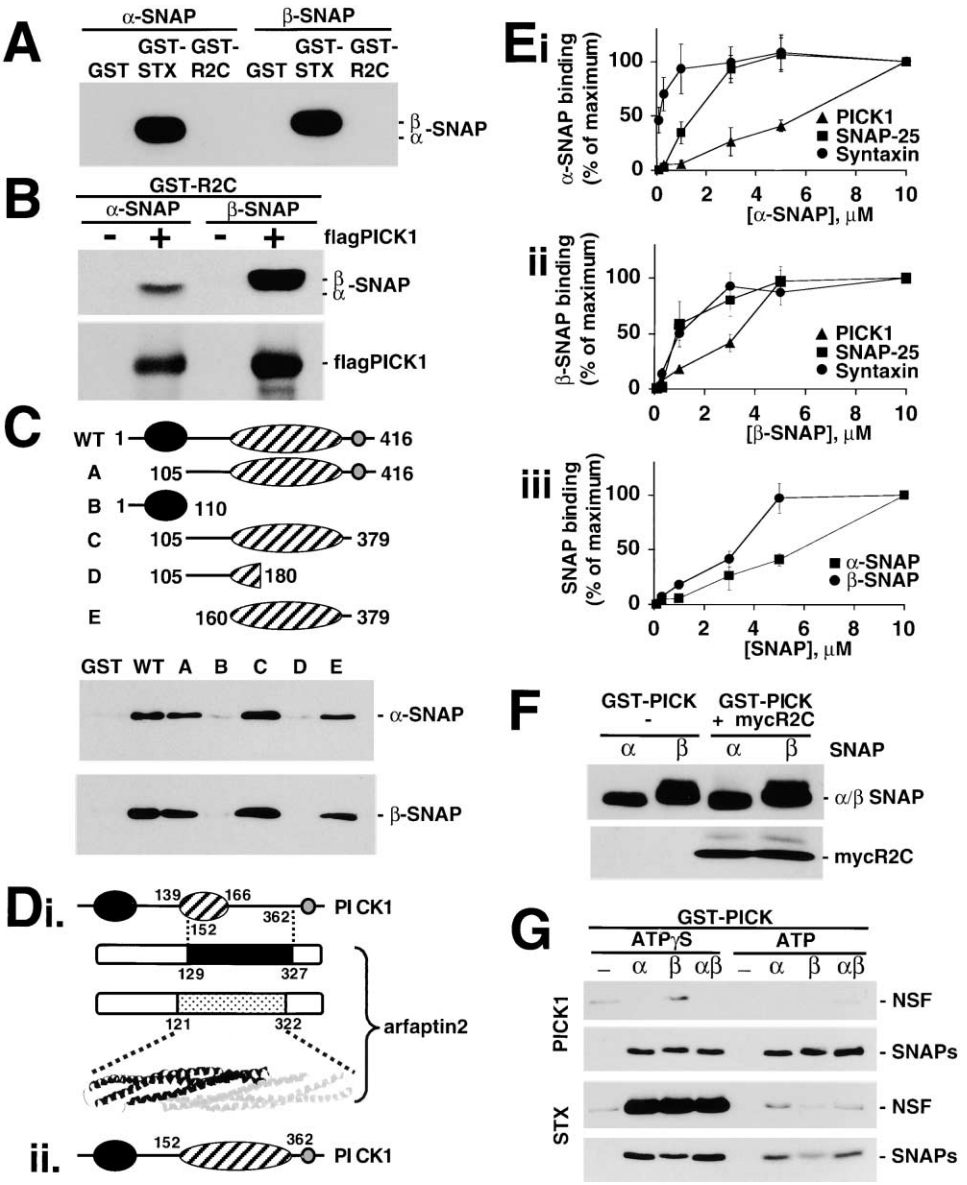

Figure 3. α-/β-SNAP Bind to PICK1

(A) SNAPs do not bind directly to GluR2C. Five micrograms GST-R2C, GST-syntaxin, or GST was immobilized on glutathione-agarose beads and incubated with 5 μM his_6α-SNAP or his_6β-SNAP in buffer B lacking nucleotide. Beads were washed thoroughly and bound SNAPs detected by Western blotting using anti-SNAP antibody.

(B) SNAPs bind to the GluR2-PICK1 complex. Two micromoles his_6flagPICK1 was bound to 5 μg GST-R2C immobilized on glutathione-agarose beads and incubated with 5 μM his_6α-SNAP or his_6β-SNAP in buffer B lacking nucleotide. Beads were washed thoroughly, and bound SNAPs and flagPICK1 were detected by Western blotting using anti-SNAP and anti-flag antibodies.

(C) SNAPs bind directly to a central region of PICK1. GST-PICK1 and truncations of GST-PICK1 (5 μg) representing the regions shown in the top panel were immobilized on glutathione-agarose beads and incubated with 2 μM his_6α-SNAP or his_6β-SNAP in buffer B lacking nucleotide. Beads were washed thoroughly and bound SNAPs were detected by Western blotting using anti-SNAP antibody.

(D) The SNAP binding site on PICK1 lies within a large coiled-coil region, predicted by homology with arfaptin 2. (i) PICK1 coiled coil domain was previously proposed to span residues 139–166 (Xia et al., 1999). PICK1 at residues 152–362 is homologous to arfaptin 2 at residues 129–327 (black shading; Takeya et al., 2000). Residues 121–322 of arfaptin 2 form a large coiled-coil region (stippled shading; Tarricone et al., 2001). (ii) Therefore, PICK1 coiled coil domain is predicted to span residues 152–362.

(E) PICK1 binds β-SNAP more strongly than α-SNAP. Both SNAP isoforms bind PICK1 less strongly than SNAREs. Five micrograms GST-syntaxin, GST-SNAP25, or GST-PICK1 was immobilized on glutathione-agarose beads and incubated with his_6α-SNAP or his_6β-SNAP in buffer B lacking nucleotide, at a range of concentrations. Beads were washed thoroughly and bound SNAPs analyzed by Western blotting using anti-SNAP antibody. Graphs show data for five experiments quantitated by scanning densitometry of Western blots. (i) α-SNAP binding to GST-syntaxin, GST-SNAP25, and GST-PICK1, (ii) β-SNAP binding to GST-syntaxin, GST-SNAP25, and GST-PICK1, (iii) α-/β-SNAP binding to GST-PICK1. Values are relative to 10 μM [SNAP] condition for each interaction.

(F) GluR2 does not contribute to the SNAP binding site. Two micrometers his_6mycR2C was bound to 5 μg GST-PICK1 immobilized on glutathione-agarose beads followed by incubation with 2 μM his_6α-/β-SNAP in buffer B lacking nucleotide. The beads were washed thoroughly,

for the PICK1-GluR2 interaction (Figure 2A). Deletion of the entire 843–852 region resulted in a large reduction in R2C-PICK1 binding, and double alanine mutations K847-V848, A849-K850, and N851-P852 significantly weakened the interaction.

To investigate whether there is a non-PDZ binding site on PICK1 that interacts with this juxtamembrane site on R2C, we assayed truncation mutants of his_6flag PICK1 for binding to wild-type GST-R2C (Figure 2B). Mutating K27-D28 to AA in the full-length protein abolished the interaction, confirming that a PDZ domain is required (Xia et al., 1999). Deleting the acidic domain (379–416) had little effect on the interaction, but further truncation to residue 180 blocked the interaction, suggesting that the coiled-coil of PICK1 is involved in GluR2 binding (see Figure 3D for explanation of PICK1 domain structure). A peptide corresponding to the "NSF region" on GluR2 (pep2m; residues 843–852) has been widely used to study the function of NSF in AMPAR trafficking (Nishimune et al., 1998; Song et al., 1998; Noel et al., 1999; Luscher et al., 1999). Although we find that the "NSF region" is also a determinant for PICK1 binding, Figure 2C shows that pep2m does not block GluR2-PICK1 interactions, suggesting that these residues do not represent the complete site for PICK1 binding, and that this peptide will not directly affect GluR2-PICK1 interactions in neuronal cultures.

These data indicate that a PDZ interaction alone is not sufficient to support full GluR2-PICK1 binding and the coiled-coil domain of PICK interacts with the "NSF region" of GluR2. This interaction can explain the formation of the complex NSF binding site described above, and suggests that NSF may regulate the GluR2-PICK1 interaction.

PICK1 Is a SNAP Receptor

The NSF cofactors α-/β-SNAP associate with GluR2 in brain (Osten et al., 1998). We demonstrate here that NSF binds R2C in an ATP-sensitive manner in the absence of SNAPs. Therefore, we investigated the role of SNAPs in this complex. Although both α-/β-SNAP bind strongly to GST-syntaxin as previously shown (Hanson et al., 1995; Hayashi et al., 1995), neither SNAP interacts with GST-R2C (Figure 3A). As recombinant α-SNAP binds to a GluR2-containing complex immunoprecipitated from brain (Osten et al., 1998), we speculated that this complex might contain PICK1, mediating the interaction of α-/β-SNAPs with R2C. Figure 3B shows that both α-/β-SNAP bind strongly to R2C-PICK1 complex preformed from purified components.

We used GST-PICK1 to assay direct PICK1-SNAP binding. Figure 3C shows that both α-/β-SNAP bind GST-PICK1. We also tested a number of GST-PICK1 truncations to determine the location of the SNAP binding site. Neither the PICK1 PDZ domain nor acidic domains are required for interaction, but SNAPs bind strongly to residues 105–379. Interestingly, this is the same region involved in the non-PDZ interaction between GluR2 and PICK1 (Figure 2). The domain structure of PICK1 has been suggested to include a small coiled-coil at residues 139–166 (Xia et al., 1999), a region that we have demonstrated to be required for PICK1 dimerization (Perez et al., 2001). PICK1 shows homology to arfaptins 1 and 2 (Takeya et al., 2000). The region of homology corresponds to residues 152–362 on PICK1, and residues 129–327 on arfaptin 2 (Figure 3Di). The crystal structure of arfaptin 2 reveals a large coiled-coil dimerization domain at residues 121–322 (Tarricone et al., 2001), which almost exactly corresponds to the region of homology with PICK1. We therefore propose a much larger coiled-coil domain for PICK1, spanning at least residues 152–362 (Figure 3Dii). In this light, the data in Figure 3C indicate SNAP binding to the coiled-coiled of PICK1. SNAPs interact with the coiled-coil regions of SNAREs, both in monomeric SNARE proteins and 20S complexes (Hayashi et al., 1995; Rice and Brunger, 1999).

We next compared the affinity of the SNAP-PICK1 interaction with the SNAP-SNARE interactions. α-SNAP binds very efficiently to syntaxin, and less strongly to SNAP-25 (Hanson et al., 1995). Figure 3E confirms this; α-SNAP binding to GST-syntaxin saturates at around 1 μM α-SNAP, and binding to SNAP-25 saturates at around 3 μM. PICK1 shows a lower affinity for α-SNAP, and does not reach saturation within this range. In contrast, β-SNAP binding to both syntaxin and SNAP-25 saturates at around 3 μM, and binding to PICK1 saturates at around 5 μM. Therefore, both SNAPs bind less strongly to PICK1 compared to SNARE proteins, and β-SNAP shows a higher level of interaction with PICK1 than α-SNAP.

Data presented in Figure 1 indicate that NSF binds more efficiently to the GluR2-PICK1 complex, compared to GluR2 alone. Pre-binding of R2C to PICK1 did not enhance SNAP interactions, indicating that PICK1 represents the full SNAP binding site in this complex (Figure 3F). Interaction of α-SNAP with SNAREs allows subsequent binding of NSF to the complex (Sollner et al., 1993; Hanson et al., 1995). However, SNAPs do not perform a similar role with PICK1, since NSF does not bind PICK1-SNAP complexes in the absence of R2C (Figure 3G). In this experiment, we used 5-fold higher concentrations of SNAP for PICK1 compared to syntaxin, so that comparable levels of SNAP would be present in the two complexes.

These data demonstrate that PICK1 has properties of a SNAP receptor. However, unlike traditional SNAREs, the PICK1-SNAP interaction is not sufficient to support NSF binding, which requires the presence of GluR2. The finding that α-/β-SNAP bind with very different affinities to PICK1 suggests distinct roles for the two isoforms.

and bound SNAPs and mycR2C analyzed by Western blotting using anti-SNAP and anti-myc antibodies.

(G) NSF does not bind PICK1-SNAP complexes. Five micrograms GST-PICK1 or GST-syntaxin (STX) was immobilized on glutathione-agarose beads followed by incubation with 2 μM $his_6\alpha$-/β-SNAP (syntaxin) or 10 μM $his_6\alpha$-/β-SNAP (PICK1) in buffer B lacking nucleotide. Where both SNAPs were used, the concentration applies to the total SNAPs. ATP/ATPγS was added, plus 50 nM his_6NSF. The beads were washed thoroughly in the nucleotide buffer, bound NSF and SNAPs were detected by Western blotting using anti-NSF and anti-SNAP antibodies.

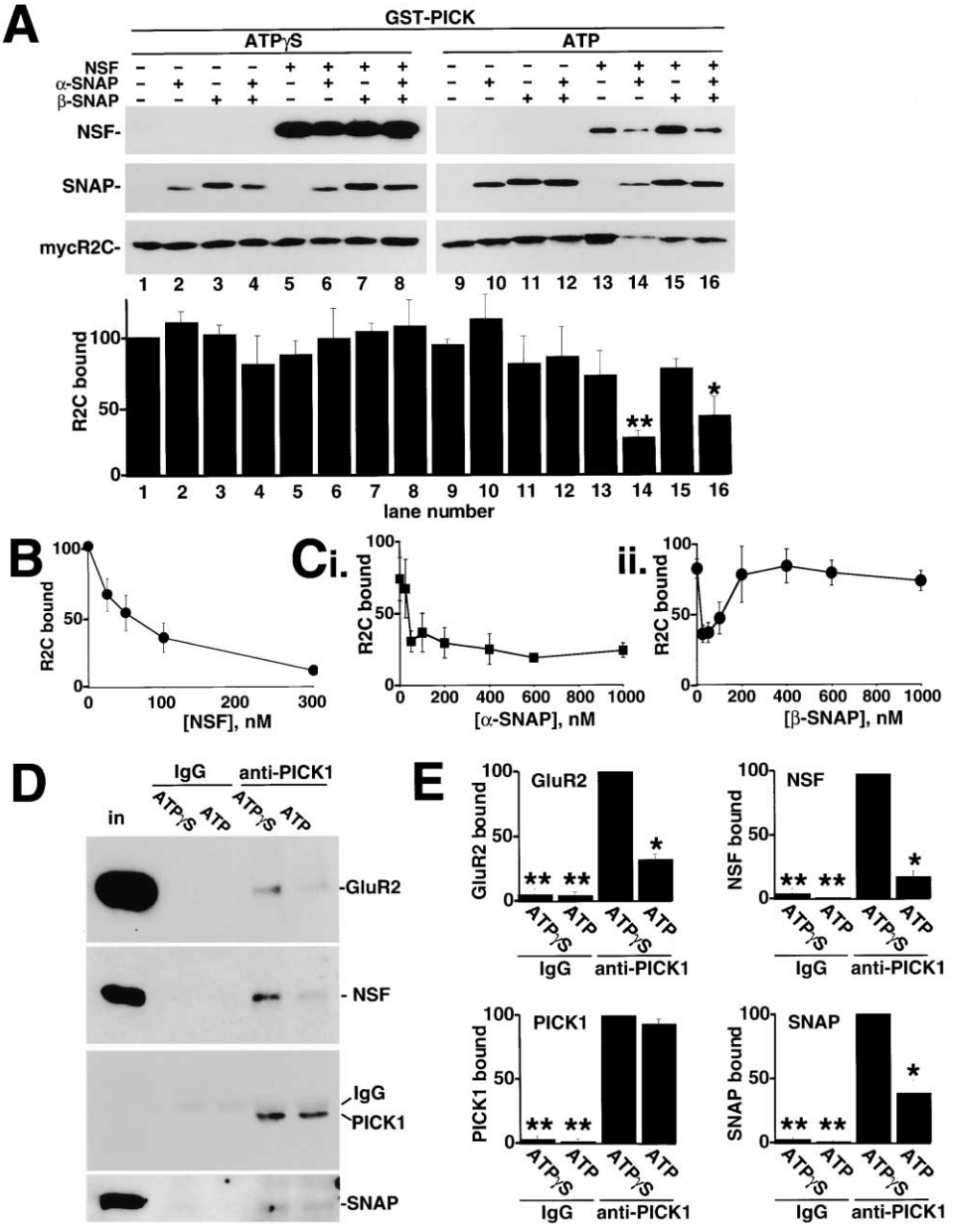

Figure 4. The ATPase Activity of NSF Disrupts the GluR2-PICK1 Complex. α-/β-SNAPs Differentially Regulate This Process

(A) NSF and SNAPs disrupt GluR2-PICK1 complex in the presence of hydrolyzable ATP. One hundred nanomoles GST-PICK1-his$_6$mycR2C complex immobilized on glutathione agarose beads was incubated with 300 nM his$_6$α-/β-SNAP and 125 nM his$_6$NSF, in buffer plus ATP/ATPγS. Beads were washed twice in buffer C and once in buffer B. Bound proteins were analyzed by Western blotting using anti-NSF, anti-SNAP, and anti-myc antibodies. Top panel shows representative Western blots for NSF, SNAPs, and mycR2C. Bottom panel shows data from four experiments, quantitated by scanning densitometry of Western blots for mycR2C. Values are relative to lane 1 ($n = 4$, t test, *$p < 0.05$, **$p < 0.001$).

(B) shows dose-response curve for NSF in disassembly of PICK1-R2C complexes. The same assay as above was carried out with different concentrations of NSF (nM) as indicated. α-SNAP was constant at 200 nM. Graph shows data from four experiments quantitated by scanning densitometry of Western blots for NSF. Values are relative to zero [NSF] condition.

(C) Differential effects of α-SNAP (i) and β-SNAP (ii) on disassembly of PICK1-R2C complexes. The same assay as above was carried out with different concentrations of SNAPs (nM) as indicated to produce dose-response curves. NSF was constant at 200 nM. Graphs show data from five experiments quantitated by scanning densitometry of Western blots for SNAPs. Values are relative to zero [NSF], zero [SNAP].

(D) GluR2, NSF, SNAPs, and PICK1 associate in an ATP-sensitive complex from hippocampus. Rat hippocampal homogenates solubilized in 1% Triton X-100 were immunoprecipitated with anti-PICK1 antibody in buffer D plus ATP/ATPγS. Bound proteins were analyzed by Western blotting using anti-GluR2/3, anti-NSF, anti-PICK1, and anti-SNAP antibodies. "in" represents 1% of the total lysate.

(E) Data from three anti-PICK1 immunoprecipitations from hippocampal lysate quantitated by scanning densitometry of Western blots as shown in (D), above ($n = 3$, t test, *$p < 0.05$, **$p < 0.005$).

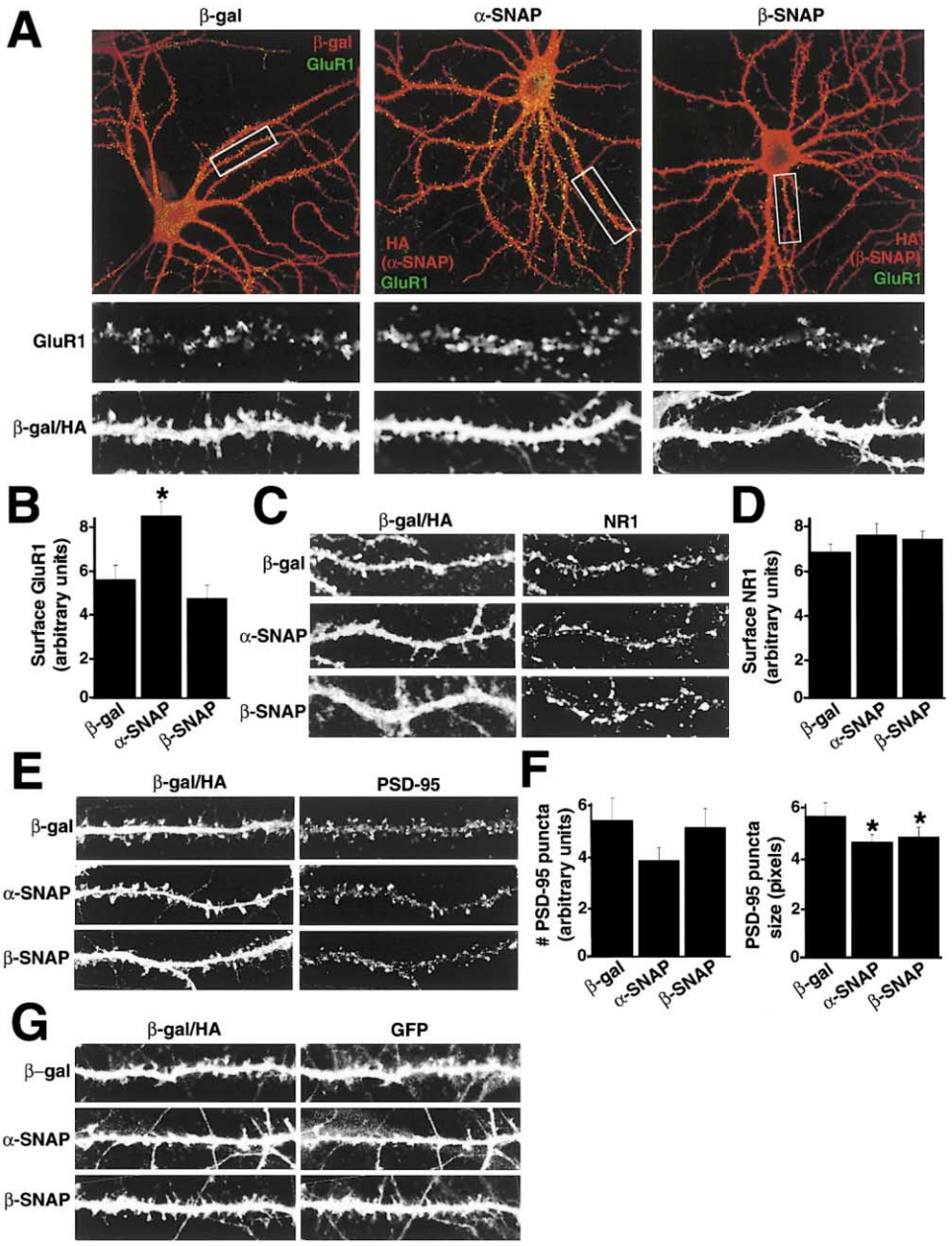

Figure 5. Overexpression of α-/β-SNAP Differentially Regulates Surface Expression of AMPA Receptors in Cultured Neurons

(A) Dissociated hippocampal neurons infected with Sindbis virus encoding β-gal, HA-tagged α-SNAP, or HA-tagged β-SNAP were stained for surface AMPAR using anti-GluR1 antibody (FITC) and for β-gal or HA-tag (Texas Red). White box defines enlargements shown in lower panels.

(B) shows quantitation of surface AMPAR on cells as shown in (A) ($n = 20$ cells, t test, $^*p < 0.001$).

(C) NMDA receptor surface expression is not significantly affected by SNAP overexpression. Hippocampal neurons infected with Sindbis virus encoding β-gal, HA-tagged α-SNAP, or HA-tagged β-SNAP were stained for surface NMDARs using anti-NR1 antibody.

(D) Quantitation of surface NMDAR on cells as shown in (C) ($n = 20$, t test).

(E) Number of postsynaptic densities is not significantly affected by SNAP overexpression. Hippocampal neurons infected with Sindbis virus encoding β-gal, HA-tagged α-SNAP, or HA-tagged β-SNAP were stained with anti-PSD-95 antibody.

(F) Quantitation of number of PSD-95 puncta per cell area in neurons as shown in (E) (left panel; $n = 20$, t test). Quantitation of average size of PSD-95 puncta in cells as shown in (E) (right panel; $n = 20$, t test, $^*p < 0.05$).

(G) Number of dendritic spines is not affected by SNAP overexpression. Hippocampal neurons coinfected with Sindbis viruses encoding GFP and either β-gal, HA-tagged α-SNAP, or HA-tagged β-SNAP were stained for β-gal or HA-tag (Texas Red).

Disruption of the GluR2-PICK1 Interaction by NSF ATPase Activity and Differential Regulation by α- and β-SNAP

By analogy with NSF/SNAP disruption of SNARE complexes, our data strongly suggest that NSF/SNAPs might disrupt GluR2-PICK1 interactions. To test this, we formed complexes of GST-PICK and his_6mycR2C, and incubated them with his_6NSF and his_6SNAPs in the presence of ATP/ATPγS (Figure 4A). NSF alone has no effect on the R2C-PICK1 interaction, despite dissociation of

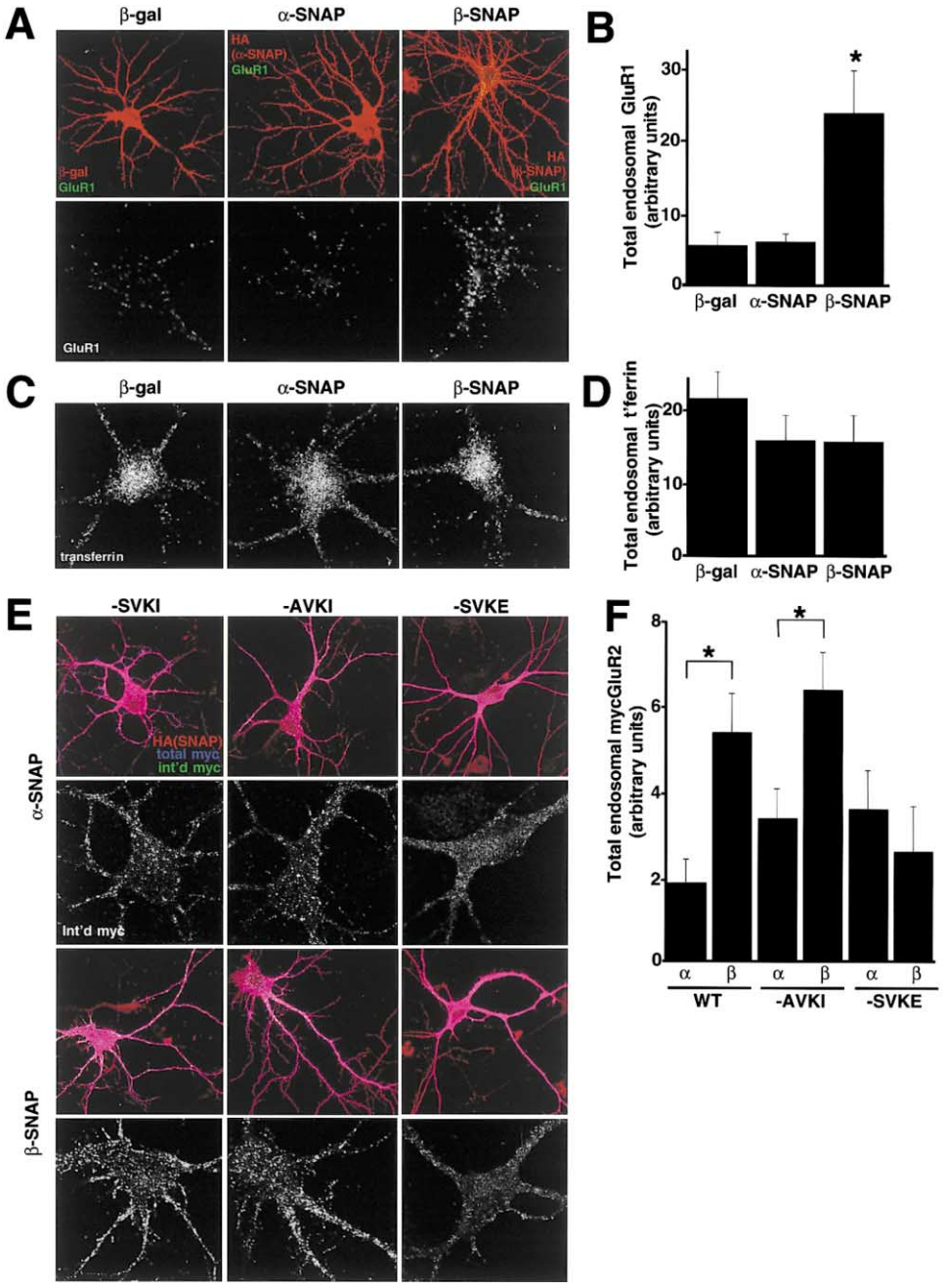

Figure 6. Overexpression of α-/β-SNAP Differentially Regulates AMPAR Endocytosis via GluR2-PICK1 Interactions

(A) Dissociated hippocampal neurons infected with Sindbis virus encoding β-gal, HA-tagged α-SNAP, or HA-tagged β-SNAP were assayed for AMPAR endocytosis by acid-stripping. Cells were stained for β-gal or HA (Texas Red) and internalized GluR1 (FITC). Bottom panels show close-ups of cell bodies for GluR1 staining.

(B) Quantitation of internalized AMPAR in cells shown in (A). Values represent total internalized GluR1 immunoreactivity normalized for cell area. ($n = 20$, t test, $^*p < 0.01$.)

(C) Overexpression of SNAPs does not affect transferrin receptor endocytosis. Hippocampal neurons infected with Sindbis virus encoding β-gal, HA-tagged α-SNAP, or HA-tagged β-SNAP were assayed for transferrin receptor endocytosis. Cells were stained for β-gal or HA (Texas Red) and internalized transferrin receptor (FITC-conjugated transferrin).

(D) Quantitation of internalized transferrin receptor in cells shown in (C). Values represent total internalized transferrin per cell area. ($n = 30$, t test.)

(E) SNAPs modulate AMPAR internalization by regulation of GluR2-PICK1 interaction. Hippocampal neurons were doubly infected with Sindbis virus encoding β-gal, HA-tagged α-SNAP, or HA-tagged β-SNAP and mycGluR2 wild-type (-SVKI), -AVKI, or -SVKE mutants. Cells were assayed for mycGluR2 endocytosis by acid-stripping. Cells were stained for β-gal or HA (Texas Red), total mycGluR2 (Cy-5), and internalized mycGluR2 (int'd myc, FITC). Upper panels show all three channels, lower panels are close-ups of cell bodies showing internalized mycGluR2.

(F) Quantitation of internalized mycGluR2 in cells shown in (E). Values represent total internalized myc immunoreactivity normalized for cell area and total myc expression level ($n = 40$, t test, $^*p < 0.01$).

NSF itself upon ATP hydrolysis (lane 13). α-/β-SNAP alone also have no effect (lanes 10–12). When both NSF and α-SNAP are added to the complex, ATP hydrolysis results in a striking dissociation of R2C from PICK1 (lane 14). α-SNAP and NSF are also released. The interaction is stable in the presence of ATPγS (lane 6), indicating that dissociation requires NSF ATPase activity. Interestingly, if β-SNAP is present instead of α-SNAP, the complex is more stable in the presence of ATP (lane 15). When both SNAP isoforms are present, disassembly of the complex is observed (lane 16), but to a lower degree of significance compared to α-SNAP alone.

Next we tested the effect of different concentrations of NSF in the disassembly assay (Figure 4B). It is important to note that the concentrations of NSF shown here refer to the monomeric protein. NSF is functional as a hexamer (Fleming et al., 1998), so the maximum concentration of the hexameric enzyme is one-sixth of the values stated. With α-SNAP constant at 200 nM, maximal disassembly of PICK1-R2C complexes is seen at around 200–300 nM NSF, which is very similar to SNARE complex disassembly (Hayashi et al., 1995). We also tested different concentrations of SNAPs in the dissociation assay, with NSF constant at 200 nM (Figure 4C). Hayashi et al. (1995) report that maximal SNARE complex disassembly requires an α-SNAP concentration of 200–400 nM. Maximal disassembly of PICK1-R2C complex occurs at around 100 nM α-SNAP, and is maintained at higher concentrations (Figure 4Ci). Interestingly, the curve for β-SNAP is markedly different (Figure 4Cii). At very low concentrations (25–100 nM), β-SNAP stimulates NSF activity to disassemble the complex. In fact, β-SNAP is more active at 25 nM (the lowest concentration tested) than α-SNAP. However, at higher concentrations (>200 nM), β-SNAP inhibits NSF and stabilizes the PICK1-R2C complex.

These data demonstrate that the ATPase activity of NSF disrupts GluR2-PICK1 interactions. The presence of either α-SNAP or very low concentrations of β-SNAP stimulates this activity. However, high levels of β-SNAP inhibit the efficacy of NSF, resulting in stabilization of the GluR2-PICK1 interaction.

Association of PICK1 with SNAPs and NSF in Brain

To confirm that interactions observed in vitro also occur in vivo, we carried out immunoprecipitations (IPs) from rat hippocampal extract (Figures 4D and 4E). IP in the presence of ATPγS with anti-PICK1 antibody, and subsequent Western blotting for SNAPs, NSF, and GluR2, show that these proteins exist as a complex in vivo. Association of PICK1 with GluR2 in brain has been demonstrated previously (Xia et al., 1999), as well as association of NSF and SNAPs with GluR2 (Osten et al., 1998). The binding of NSF and SNAPs to the in vivo complex is sensitive to ATP hydrolysis, and GluR2 dissociates from PICK1 in the presence of MgATP, demonstrating that the ATPase activity of NSF disassembles native PICK1-GluR2 complexes from brain. This experiment demonstrates that NSF and SNAPs are associated with PICK1-containing complexes in vivo, and that the regulation of the PICK1-GluR2 interaction by NSF ATPase activity and SNAPs we have described in vitro also occurs in vivo.

Differential Effects of α- and β-SNAP on Trafficking of AMPA Receptors in Neurons

The PICK1-GluR2 interaction functions in endocytosis of AMPARs from the synaptic plasma membrane (Iwakura et al., 2001; Kim et al., 2001; Perez et al., 2001; Xia et al., 2001). We hypothesized that the NSF-GluR2 interaction stabilizes surface AMPARs by disrupting PICK1-GluR2 complexes, preventing PICK1-dependent endocytosis of AMPARs. The differential effects of α-/β-SNAP at high concentration provided a test for this hypothesis. α-/β-SNAP were overexpressed in dissociated hippocampal cultures and trafficking of AMPARs was analyzed. As α-/β-SNAP have similar activities in SNARE-mediated exocytosis (Hayashi et al., 1995; Sudlow et al., 1996), both isoforms should increase surface expression of recycling plasma membrane proteins. Therefore, a differential effect of α-/β-SNAP in this assay would be consistent with differential SNAP regulation of NSF-dependent disruption of GluR2-PICK1 interactions. Our data suggest that high levels of β-SNAP will inhibit NSF-mediated disruption of GluR2-PICK1 complexes, resulting in increased receptor internalization. In contrast, α-SNAP will enhance NSF-mediated dissociation of PICK1 from GluR2, stabilizing the receptor at the cell surface.

We overexpressed HA-tagged α-/β-SNAP in dissociated hippocampal cultures using sindbis virus, and assayed surface AMPARs by immunocytochemistry (Figure 5). Cells expressing exogenous α-SNAP, β-SNAP, or β-galactosidase (β-gal) as a control show normal morphology (Figure 5A). α-SNAP overexpression increased surface AMPAR staining by 50% compared to β-gal control, whereas β-SNAP overexpression results in a lower level of surface AMPAR, similar to control (Figures 5A and 5B). The intensity of HA staining for α-/β-SNAP infections was the same. To demonstrate specificity of these results for AMPAR, we analyzed surface staining for NMDAR NR1 subunit. Figures 5C and 5D show that surface levels of NMDAR are the same when either SNAP is overexpressed. To test whether the size or total number of postsynaptic sites altered with SNAP overexpression, we analyzed the scaffold protein PSD-95 (Figures 5E and 5F). The number of PSD-95 puncta is unaffected, and the average puncta size is slightly reduced for both α-/β-SNAP overexpression compared to β-gal control. This reduction occurs by an unknown mechanism, but the crucial observation is that both SNAPs produce the same effect. As a control for spine number, we used sindbis viruses to coexpress GFP and SNAP/β-gal in the same neurons. Although SNAPs and β-gal are diffusely distributed throughout the cell, GFP can be used as an independent means of visualizing spines. Figure 5G shows that the density of dendritic spines is the same when either SNAP is overexpressed.

We investigated the effects of SNAP overexpression on AMPAR endocytosis by acid-stripping experiments (Carroll et al., 1999; Beattie et al., 2000; Iwakura et al., 2001). Figure 6A shows that α-SNAP overexpression has no effect on AMPAR endocytosis compared to control, whereas β-SNAP enhances receptor internalization 4-fold (Figure 6B). This is consistent with the biochemical data presented here, which indicates that high levels of β-SNAP inhibit NSF-mediated disassembly of GluR2-PICK1 complexes. In cells overexpressing β-SNAP,

GluR2-PICK1 interactions are stabilized, allowing increased PICK1-mediated endocytosis of AMPARs. As a control, we analyzed internalization of the transferrin receptor. There is no significant difference in internalization of transferrin receptor when either α-/β-SNAP is overexpressed, (Figures 6C and 6D) demonstrating that endocytosis per se is unaffected by β-SNAP.

To provide further evidence that the observed effect of SNAPs on AMPAR trafficking is acting upon GluR2-PICK1 complexes, we studied the internalization of myc-tagged GluR2 C-terminal mutants coexpressed with SNAPs. Mutation of the C-terminal isoleucine of GluR2 (-SVKE) abolishes binding of GluR2 to ABP, GRIP, and PICK1 (Osten et al., 2000). A more selective mutation, -AVKI, allows interaction with PICK1, but not with ABP/GRIP (Osten et al., 2000). If SNAP overexpression affects AMPAR trafficking via GluR2-PICK1 interactions, then α-/β-SNAP should only differentially affect endocytosis of wild-type (-SVKI) and the -AVKI mutant, whereas -SVKE should show the same behavior with both SNAPs. If the observed effects on endogenous AMPAR trafficking are due to nonspecific mechanisms such as SNARE complex disassembly, then all three mycGluR2 mutants should behave similarly with α-/β-SNAP. Figures 6E and 6F demonstrate that mycGluR2 that can bind PICK1 (-SVKI, -AVKI) show a significantly higher level of endocytosis with β-SNAP coexpression, compared to α-SNAP. However, mycGluR2 that cannot bind PICK1 (-SVKE) shows the same level of receptor endocytosis with both α-/β-SNAP. Specifically, disrupting GluR2-ABP/GRIP interactions (compare -SVKI with -AVKI) does not abolish the differential modulation of receptor endocytosis by α-/β-SNAP. This experiment strongly suggests that overexpression of α-/β-SNAP differentially regulates GluR2-PICK1 interactions to control AMPAR endocytosis. Therefore, NSF-mediated disassembly of GluR2-PICK1 complexes, which we have shown to be differentially regulated by α-/β-SNAP in vitro, occurs in living neurons and is involved in controlling AMPAR trafficking.

Discussion

In this study, we demonstrate that the ATPase activity of NSF disrupts GluR2-PICK1 interactions. α-/β-SNAP differentially modulate this activity. This report demonstrates NSF-mediated disassembly of a protein complex other than the SNAREs.

Molecular Interactions in the GluR2-PICK1-NSF-SNAP Complex

There are a number of striking differences between the protein interactions described here for GluR2-PICK1-NSF-SNAPs and those in 20S complexes. NSF will not bind SNAREs in the absence of SNAPs (Sollner et al., 1993; Hanson et al., 1995). In contrast, SNAPs are not required for binding of NSF to GluR2-PICK1 complexes. Indeed, NSF will not bind to PICK1-SNAP complexes in the absence of GluR2; GluR2 must be present for NSF to bind. SNAPs have been shown to be required for stimulation of NSF ATPase activity (Barnard et al., 1997). Here we show that NSF binding to both GluR2 and GluR2-PICK1 complexes is highly sensitive to ATP hydrolysis in the absence of SNAPs, indicating that interaction with GluR2 is sufficient to stimulate the enzymatic activity of NSF. However, we demonstrate that both α-/β-SNAP bind to PICK1, and that they are required for efficient complex disassembly. As SNAPs bind to PICK1 and also to NSF, it is likely that they function to transfer the mechanical force produced by ATP hydrolysis from NSF to PICK1. This has been suggested for disassembly of 20S complexes by NSF and α-SNAP (Rice and Brunger, 1999). It has been shown that NSF drastically changes conformation on ATP hydrolysis (Hanson et al., 1997). When this occurs, SNAPs could act as lever arms, forcing PICK1 away from GluR2 and disrupting the non-PDZ interaction. We show that mutations in the "NSF binding region" of GluR2 weaken PICK1-GluR2 interactions, so it follows that dissociation by NSF at this binding site would also weaken PICK1-GluR2 binding.

SNAPs bind to regions on SNARE proteins proposed to be coiled-coils, which form the helical bundle of the 7S complex (Hayashi et al., 1995; Rice and Brunger, 1999). PICK1 has a putative coiled-coil domain that is a site for dimerization (Xia et al., 1999; Perez et al., 2001). Homology of PICK1 with the arfaptin 2 coiled-coil domain suggests that the coiled-coil of PICK1 is larger than previously suggested (Takeya et al., 2000; Tarricone et al., 2001; this study). The minimal binding site for SNAPs on PICK1 fits very well with the newly proposed coiled-coil. The crystal structure of the arfaptin 2 coiled-coil indicates that as a dimer, it forms a helical bundle with each monomer contributing three α helices (Tarricone et al., 2001). It is likely that PICK1 dimers have a similar structure. Crystal structure studies have suggested that SNAPs recognize general three-dimensional surface features of the SNARE bundle rather than specific residues (Rice and Brunger, 1999). SNAPs may recognize the proposed helical bundle of PICK1 and the helices of the SNARE complex in a similar way. In the 20S complex, NSF is thought to exert its disassembling effect on SNAREs via SNAPs by rotational shearing of the helical bundle (May et al., 1999). It is possible that NSF and SNAPs induce such a shearing movement to disrupt the interaction between the PICK1 coiled-coil and GluR2.

Specific functions for β-SNAP have been difficult to elucidate, and it has been demonstrated that α-/β-SNAP are equivalent interchangeable isoforms with the same activity in calcium-regulated exocytosis in chromaffin cells (Sudlow et al., 1996) and SNARE complex assembly-disassembly reactions in vitro (Wilson et al., 1992; Hayashi et al., 1995). β-SNAP has been shown to bind synaptotagmin (Schiavo et al., 1995), so it has been implicated as playing a specific role in the regulation of synaptic vesicle exocytosis, although further physiological evidence for this has not been provided. Here we show that β-SNAP binds PICK1 much more strongly than α-SNAP, identifying PICK1 as a specific β-SNAP binding partner. Although β-SNAP can substitute for α-SNAP in dissociation of GluR2-PICK1 complexes, it has a biphasic concentration dependence. At very low concentrations, it stimulates NSF-mediated disruption of PICK1-GluR2 more efficiently than α-SNAP, and at higher concentrations, it inhibits this activity. Under these inhibitory conditions, NSF also remains bound in

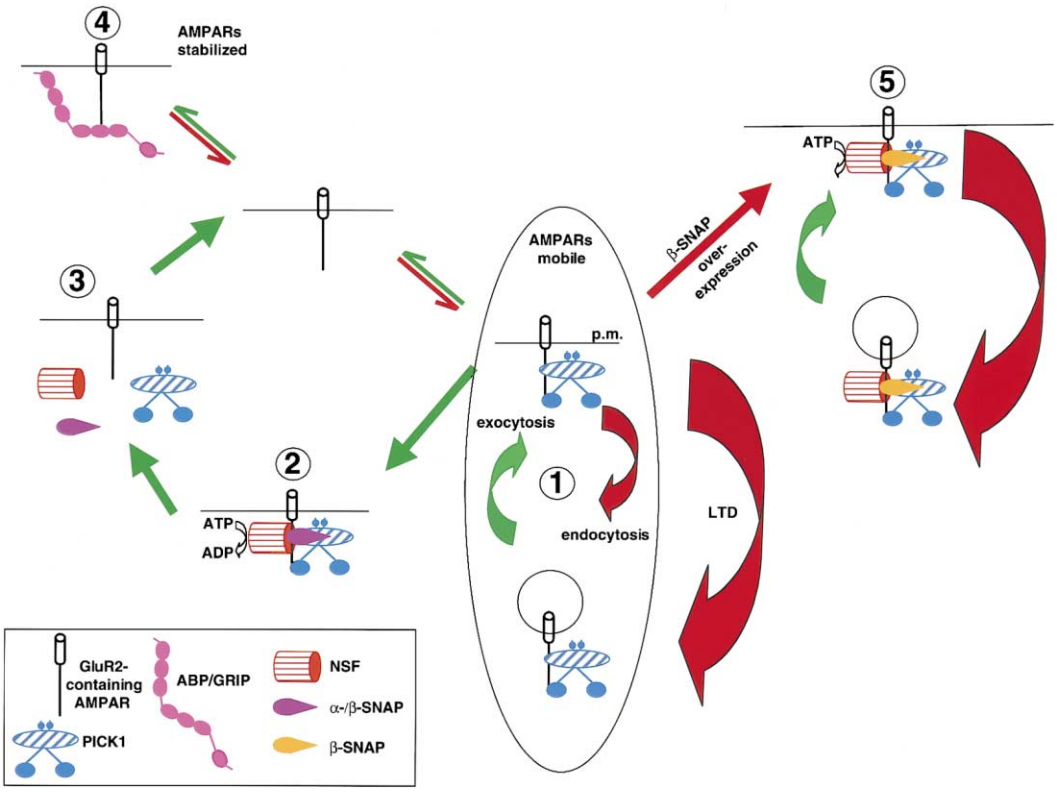

Figure 7. Models of NSF/SNAP-Mediated Regulation of PICK1-AMPAR Interactions in AMPAR Cycling and LTD

GluR2-containing AMPARs that are bound to PICK1 are mobile and contained in a cycling receptor pool contributing to endocytosis from the plasma membrane, and insertion into the plasma membrane by exocytosis (1). NSF ATPase activity, stimulated by low levels of SNAP binding (2), will disassemble a proportion of GluR2-PICK1 complexes (3), allowing anchorage of some of the cycling receptors by ABP/GRIP at the plasma membrane (4). The remainder of GluR2-containing receptors may remain bound to PICK1, resulting in entry into the recycling pathway. Inhibition of NSF activity under physiological circumstances, possibly by regulation of SNAP binding, results in increased stability of PICK1-GluR2 complexes, and consequent increase in endocytosis manifested as LTD. This is likely to occur in conjunction with phosphorylation of GluR2 at S880, which blocks binding of ABP/GRIP, but permits binding of PICK1 (Matsuda et al., 1999; Chung et al., 2000).
Overexpression of β-SNAP inhibits the NSF-mediated disruption of GluR2-PICK1 interactions, resulting in enhanced AMPAR internalization (5).
Straight arrows represent biochemical events, curved arrows AMPAR trafficking. Red shows events leading to downregulation of AMPARs at synapses, green arrows show upregulation of receptors.

the presence of ATP, suggesting that β-SNAP can block the ATPase activity of NSF. It is possible that the extent of PICK1-SNAP binding, or the SNAP isoform present in the complex, is regulated in vivo. Such regulation need not depend on the absolute or relative concentration of SNAP isoforms in the cell, but may involve phosphorylation or binding of other protein components. This could in turn regulate the activity of NSF in the complex, and provide a potential means of controlling GluR2-PICK1 interactions, and hence AMPAR trafficking.

In summary, we propose a mechanism in which GluR2, PICK1, NSF, and SNAPs form a complex. NSF, tethered to GluR2, delivers ATP-driven rotational force to PICK1 via SNAPs, resulting in dissociation of the complex.

Significance to AMPA Receptor Trafficking

We recently reported a role for ABP/GRIP in stabilizing AMPARs at the synapse (Osten et al., 2000). Phosphorylation of GluR2 at serine 880 disrupts interactions with ABP/GRIP (Matsuda et al., 1999; Chung et al., 2000; Matsuda et al., 2000). PICK1 binding is insensitive to S880 phosphorylation, so PICK1 can bind to the unanchored receptor to permit endocytosis (Chung et al., 2000; Matsuda et al., 2000). It has been demonstrated that an interaction between GluR2 and PICK1 is a requirement for AMPAR endocytosis and some forms of LTD (Iwakura et al., 2001; Kim et al., 2001; Xia et al., 2001). The GluR2-NSF interaction is required for maintained surface expression of AMPARs, as blockade of this interaction using a specific binding-site peptide (pep2m) results in a run-down of AMPAR currents (Nishimune et al., 1998; Song et al., 1998; Noel et al., 1999; Luscher et al., 1999). This run-down occludes LTD, suggesting that LTD and rundown after blockade of GluR2-NSF interaction employ the same mechanisms (Luthi et al., 1999). LTD is at least partly expressed by removal of AMPARs from the synapse by endocytosis (Luscher et al., 1999; Beattie et al., 2000; Matsuda et al., 2000; Wang and Linden, 2000). Furthermore, inhibition of NSF activity by NEM enhances agonist-induced endocytosis of AMPARs (Luscher et al., 1999). Taken together, these reports suggest that the function of the GluR2-NSF interaction is to inhibit AMPAR endocytosis. Our demonstration that NSF can regulate the GluR2-PICK1 interaction

provides a molecular mechanism for these observations. The run-down after treatment with pep2m can therefore be explained in terms of blockade of the disassembling activity of NSF on GluR2-PICK1 interactions. This allows more PICK1 to bind GluR2, resulting in enhanced AMPAR internalization.

We used SNAP overexpression to demonstrate that the disassembly of GluR2-PICK1 complexes by NSF observed in vitro also occurs in living neurons. The addition of high concentrations of β-SNAP to the NSF-mediated disassembly assay in vitro stabilizes the PICK1-R2C complex. Overexpression of β-SNAP in neurons enhances AMPAR endocytosis, which is consistent with a stabilized GluR2-PICK1 interaction. Furthermore, mutagenesis of the GluR2 C terminus indicates that this effect is induced only on GluR2 that is able to bind PICK1 (wild-type and -AVKI mutant). The -SVKE mutant, which cannot interact with PICK1, shows the same level of endocytosis on overexpression of both SNAP isoforms. This demonstrates that the enhanced endocytosis induced by β-SNAP overexpression is specifically exerted on GluR2-PICK1 complexes, and therefore that NSF/SNAPs regulate AMPAR endocytosis via GluR2-PICK1 interactions in living neurons. It is possible that as yet unidentified PDZ proteins interact with the C terminus of GluR2, and that such proteins might show similar profiles of binding to the C-terminal mutants as PICK1. In this case, the data shown in Figures 6E and 6F do not exclude the possibility that mycGluR2 endocytosis is affected by SNAP overexpression when interacting with a protein other than PICK1.

High concentrations of α-SNAP enhanced NSF-mediated disassembly of PICK1-R2C complexes in vitro. Interestingly, α-SNAP overexpression does not reduce receptor endocytosis below control levels, suggesting that NSF is highly active on GluR2-PICK1 complexes at steady state, presumably due to optimum levels of endogenous SNAP binding. α-SNAP stimulates NSF-mediated disassembly of GluR2-PICK1 complexes across a wide range of concentrations in vitro (Figure 4C), so it follows that α-SNAP overexpression in neurons would have little effect on these complexes. The striking rundown of AMPAR currents observed by others after blocking GluR2-NSF interactions also supports the notion that NSF has a high basal activity on AMPARs (Nishimune et al., 1998; Song et al., 1998; Luscher et al., 1999; Noel et al., 1999). However, a basal level of AMPAR endocytosis is seen in both control neurons and α-SNAP overexpressing cells, suggesting that a proportion of GluR2-PICK1 interactions remain intact.

It might be expected that the larger pool of AMPAR available at the plasma membrane observed with α-SNAP overexpression would lead to enhanced endocytosis. However, we demonstrate an unchanged level of AMPAR endocytosis. When related to the size of the available pool of receptor on the plasma membrane, this could be interpreted as a reduction in the rate of endocytosis. In this case, α-SNAP overexpression results in a small stimulation of NSF-mediated disassembly of GluR2-PICK1 complexes, which is consistent with our model.

The increased surface AMPAR induced by overexpressed α-SNAP that we observe could be explained by enhanced SNARE-mediated exocytosis of vesicles carrying AMPAR to the plasma membrane (Lledo et al., 1998; Lu et al., 2001). α-/β-SNAP have similar activities in regulation of exocytosis and SNARE complex disassembly (Hayashi et al., 1995; Sudlow et al., 1996). The lower level of surface AMPARs on overexpression of β-SNAP (similar to control levels) can be explained in terms of an equilibrium between two processes. Enhanced SNARE complex disassembly results in increased plasma membrane insertion of AMPAR, and β-SNAP-stabilized GluR2-PICK1 interactions lead to increased AMPAR internalization.

AMPAR trafficking is thought to involve constitutive cycling of receptors by endocytosis/exocytosis, as well as regulated events as part of LTD (endocytosis) and LTP (exocytosis). AMPAR endocytosis during some forms of LTD is dependent upon GluR2 phosphorylation and regulation of accessory protein binding (Matsuda et al., 2000; Iwakura et al., 2001). The NSF-mediated disassembly of the GluR2-PICK1 complex we have described in this study is therefore likely to be crucial in limiting endocytosis of AMPARs to maintain constitutive cycling at a constant rate and hence maintain a constant level of receptors at the synaptic membrane. This mechanism is depicted in Figure 7. From this baseline, LTD could be induced (in conjunction with phosphorylation events) by reducing the activity of NSF, possibly by modulation of SNAP-PICK1 binding, to stabilize GluR2-PICK1 interactions, and consequently enhance receptor endocytosis.

We have identified the molecular mechanism for the activity of NSF in AMPA receptor trafficking, and demonstrated that NSF can function as a disassembling molecular chaperone in a protein complex other than the 20S particle. As additional NSF binding partners are identified, it is possible that this ATPase, previously thought to be faithful to the SNARE complex, will show more promiscuous chaperone behavior.

Experimental Procedures

Plasmids and Plasmid Construction

His_6NSF, his_6α-/β-SNAP were expressed from pQE9 plasmids (Qiagen). His_6flagPICK1, his_6mycR2C were cloned by PCR and ligation into pPROExHT (GibcoBRL). GST-R2C and mutants were previously described (Osten et al., 1998). GST-PICK1 was cloned by PCR and ligation into pGEX-4T1 (Pharmacia). Hemaglutinin (HA)-tagged sindbis virus plasmids encoding α-/β-SNAP were cloned by PCR with 5′ primers incorporating HA and ligation into pSinRep5 (Invitrogen).

Buffers

A: 25 mM HEPES-KOH (pH 7.3), 150 mM KCl, 1% Triton X-100, 10% glycerol, 1 mM DTT.
B: 25 mM HEPES-KOH (pH 7.3), 150 mM KCl, 3 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT.
C: 25 mM HEPES-KOH (pH 7.3), 400 mM KCl, 3 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 1 mM DTT.
D: 25 mM HEPES-KOH (pH 7.8), 150 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT.

Binding reactions were in 1 mM ATP, ATPγS, or the absence of nucleotide as designated.

Expression and Purification of Recombinant Proteins

His_6NSF, his_6SNAP were expressed in M15[pREP4] (Qiagen). His_6flagPICK1, his_6mycR2C were expressed in DH5α (Gibco). Purification of his_6 proteins was performed as described (Hanson et al.,

1995). GST-fusions expressed in BL-21 (Stratagene) were purified as described (Osten et al., 1998).

His_6SNAP Binding

GST-fusions (5 μg) were immobilized on glutathione-agarose beads (GAB) in 0.5 ml buffer A for 1 hr. After two washes in buffer B, beads were incubated with his_6α-/β-SNAP for 2 hr. After four washes with buffer B, bound proteins were detected by Western blotting using Cl 77.2 anti-SNAP monoclonal (Synaptic Systems).

His_6NSF Binding

GST-fusions (5 μg) were immobilized on GAB in buffer A. After two washes in buffer A plus 1 mM nucleotide, beads were incubated with 10 nM his_6NSF for 1 hr. After four washes with buffer A (plus nucleotide), bound proteins were detected by Western blotting using R32 anti-NSF polyclonal (Osten et al., 1998). Peptide blocking experiments utilized pep2m and pep4c as described (Nishimune et al., 1998). Peptides were preincubated with 10 nM his_6NSF or his_6flagPICK1 for 1 hr in buffer A plus ATPγS, followed by incubation with 5 μg GST-R2C immobilized on GAB for 1 hr in the same buffer.

His_6mycR2C/flagPICK1 Binding

GST-fusions (5 μg) were immobilized on GAB in buffer A. After two washes in buffer A, beads were incubated with his-tagged protein at 2 μM (saturating levels) or 10 nM for his_6flagPICK1 AA mutants. For further binding of SNAP/NSF, beads were treated as described above. Beads were finally washed four times in buffer B. For GST-R2C AA mutant experiment, final washes were twice in buffer C followed by twice in buffer A. Bound proteins were detected by Western blotting using 9E10 monoclonal anti-myc (Santa Cruz) or M2 anti-flag (Sigma).

Formation and Disruption of PICK-R2C-NSF-SNAP Complex

Five micrograms GST-PICK was immobilized on GAB in buffer A. After two washes in buffer B, beads were incubated with 100 nM his_6mycR2C for 1 hr. After washing three times with buffer B (ATP/ATPγS), beads were incubated with his_6α-/β-SNAP and his_6NSF in buffer B (ATP/ATPγS) for 1.5 hr. Beads were finally washed twice with buffer C (ATP/ATPγS), then once with buffer B (ATP/ATPγS). Proteins were detected by Western blotting using 9E10 anti-myc (Santa Cruz) R32 anti-NSF, or Cl 77.2 anti-SNAP (Synaptic Systems).

Coimmunoprecipitation from Rat Hippocampal Extract

This was performed essentially as described (Osten et al., 1998). Hippocampi from 8- to 12-week-old rats were homogenized in buffer D plus ATP/ATPγS plus protease inhibitors. TX-100 was added to 1%, for 20 min. Extracts were centrifuged at 100,000 × g for 20 min. One milligram of this extract was used per condition. Ten micrograms N-18 anti-PICK1 antibody (Santa Cruz) was added for 2–3 hr, followed by protein G agarose for 1 hr. Finally, beads were washed 3–5 times with buffer D (ATP/ATPγS, plus 1% TX-100), and proteins were detected by Western blotting using AB1506 anti-GluR2/3 (Chemicon), R32 anti-NSF, N-18 anti-PICK1 (Santa Cruz), or Cl 77.2 anti-SNAP (Synaptic Systems).

Quantitation of Western Blots

To establish significant differences between conditions in biochemical experiments, films of Western blots from at least three identical experiments obtained by Enhanced Chemiluminescence (ECL) were scanned and analyzed using NIH Image 1.62. One condition was designated 100%, and all other values taken as a percentage of this. This is used to demonstrate significant differences between pairs of conditions, rather than absolute levels of binding in a complex. Error bars are standard errors, and t tests were carried out to determine significant differences.

In experiments where multiple proteins were analyzed by Western blotting, samples were split and separate blots carried out for each protein.

Sindbis Virus Infections

BHK cells were electroporated with RNA of pSinRep5-α-SNAP/β-SNAP/β-gal/GFP/mycGluR2 mutants (Osten et al., 2000) and the helper DH(26S) according to Sindbis Expression System manual (Invitrogen). Virus production and infections were carried out as described (Osten et al., 2000).

Immunocytochemistry

Hippocampal primary neurons were prepared from E18 SD rat tissue as described (Osten et al., 2000). Live immunostaining: anti-GluR1 N terminus (Calbiochem, 4 μg/ml), anti-NR1 extracellular loop (54.1; Siegel et al., 1994, 10 μg/ml), anti-myc 9E10 (Santa Cruz, 4 μg/ml) were diluted in conditioned NB-B27 medium and incubated with cells for 15 min at 37°C. Cells were washed, fixed, permeabilized, and immunostained as described (Osten et al., 2000). Fixed immunostaining: primary antibodies: goat anti-HA (Santa Cruz, 8 μg/ml), anti-HA (Covance, 0.25 μg/ml), anti-β-gal (Sigma, 1 μg/ml), anti-β-gal (0.25 μg/ml, Chemicon), anti-PSD-95 (Upstate Biotechnology). Secondary antibodies were from Jackson ImmunoResearch (dilution 1:300). Transferrin receptor was detected using FITC-conjugated transferrin (20 μg/ml, Molecular Probes).

Acid-stripping experiments were performed essentially as described (Carroll et al., 1999). Live cells were labeled with anti-GluR1, anti-myc 9E10, or FITC transferrin for 15 min at 37°C. Cells were washed twice in PBS and returned to conditioned medium for a further 15 min at 37°C. Surface antibody was stripped away using 200 mM acetic acid, 500 mM NaCl in PBS. Cells were fixed, permeabilized, and stained for HA-SNAP, β-gal, or total mycGluR2.

Images were acquired on a Nikon PCM 2000 confocal microscope, and analyzed using Compix Imaging Systems software. To analyze total surface expression of GluR1/NR1, cell outlines were defined as the extent of HA-SNAP/β-gal staining, and total intensity of GluR1/NR1 staining was measured in this area. The ratio of surface signal:cell area was calculated. To analyze levels of internalization, antibody-stained subunit in internal vesicles was imaged by thresholding minimal object size and intensity. The total staining for myc, GluR1, or transferrin in all vesicles for a given cell was measured. For endogenous AMPAR, the ratio of internalized signal:cell area was calculated. For mycGluR2, the value was also normalized for total mycGluR2 expression. In a given experiment, data for at least 20 cells were collected, error bars defined as standard errors, and t tests performed to determine significant differences. To analyze number and size of PSDs, infected neurons were stained for HA-SNAP or β-gal and PSD-95. PSD-95 staining in PSDs was imaged by thresholding minimal object size and intensity. The number of PSDs was then determined within the cell area, and the number:cell area was calculated. The size of PSDs was determined by calculation of the average puncta size per neuron. In each case, "n" refers to the number of cells analyzed in a given experiment.

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